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Short term, low dose fluoxetine prevents estrous cycle-linked increase in anxiety-like behaviour in female rats.

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Abstract

Background and Aims

We sought a robust behavioural test that evoked increased anxiety-like behaviour during the late diestrus phase of the estrous cycle (similar to the premenstrual period in women) and tested whether this could be prevented by acute low-dose fluoxetine.

Methods

Female Wistar rats in different stages of their cycle were exposed to 4 different tests of anxiety-like behaviour.

Results

No estrous cycle differences were detected in fear potentiated startle or conditioned freezing to an aversive context. In a light switch-off test where rats move from one compartment of a shuttle-box to the other to turn off an aversive light, females displayed enhanced responding in late diestrus. During isolation restraint stress females in late diestrus emitted 3 times more 22 kHz ultrasound vocalizations (USV) than at other cycle stages. Using the USV test, short-term administration of low dose fluoxetine (1.75mg Kg^{-1}, i.p.) designed to blunt the sharp fall in brain allopregnanolone concentration during late diestrus but without affecting 5-HT systems, prevented the increase in isolation stress-evoked USVs.

Conclusions

The light switch-off and isolation restraint-induced USV tests evoke unconditioned adverse emotional responses that are ethologically relevant and sensitive to estrous cycle stage. The USV test fulfils many criteria required of a model for premenstrual syndrome in women. Using the USV test short term administration of fluoxetine to increase brain allopregnanolone concentration without affecting 5-HT systems, prevented the increased USV responding in late diestrus. Short-term low dose fluoxetine treatment may have potential to alleviate development of adverse premenstrual symptoms in women.

Keywords: estrous cycle, fear, anxiety, startle, freezing, ultrasonic vocalisations; fluoxetine; female rat

Introduction

In women, premenstrual syndrome (PMS) and its extreme form premenstrual dysphoric disorder (PMDD) is by far the most common female mental health disturbance, afflicting up to 80% of women of reproductive age (approximately 15-45 years old) (Tschudin et al, 2010). PMS is characterized by adverse psychological symptoms including anger, irritability, mood swings and anxiety that typically develop each month during the luteal phase of the menstrual cycle (Halbreich et al, 2007). The condition is not well managed. Patients are advised on diet and lifestyle changes; and may be prescribed antidepressants – usually an SSRI (Imai et al, 2015; Ismaili et al 2016). Whilst these can be helpful in reducing symptoms, side effects are not uncommon, particularly after long term treatment (Majoribanks et al, 2013). Hormonal contraceptives that stabilise hormone levels are effective in some women, but symptoms persist in others, especially during the drug free period if intermittent dosing regimens are followed (Baker and O'Brien, 2012). In addition, the associated loss of fertility may be unwanted.

During the late luteal phase, secretion of progesterone decreases rapidly. We found recently that women who develop PMS experienced a much sharper decline in progesterone towards the end of their late luteal phase, when symptoms are at their worst, compared to asymptomatic women in whom progesterone declined gradually (Lovick et al, 2017). Measures to slow the rate of decline of progesterone during the luteal phase could therefore be a potential strategy to prevent the development of symptoms.

In the female rat progesterone secretion declines rapidly during the late diestrus phase of the cycle (Butcher et al, 1975). Several studies have shown that a rapid decline in progesterone, and hence is neuroactive

metabolite allopregnanolone (ALLO) triggers upregulation of extrasynaptic $GABA_A$ receptor subunit expression, increased excitability of neural circuits involved in mediating anxiety-related behaviours and an increase in anxiety-like behaviour (Griffiths et al, 2005; Smith et al, 1998; Devall et al, 2009). Progesterone withdrawal has been adopted as a model of premenstrual dysphoria by several laboratories (e.g. Gallo and Smith, 1993; Li et al, 2012; Löfgren et al, 2009; Smith et al, 2006). Interestingly, the "withdrawal" effect of a sharp decline in progesterone was absent in rats in which progesterone concentration fell gradually (Doornbos et al, 2006). Based on these findings we hypothesized that measures to blunt the rapid fall in progesterone and hence ALLO during late diestrus should be able to prevent the behavioural disturbance.

There have been few studies attempting to model premenstrual dysphorias in rats undergoing spontaneous cycles. However, increased aggression towards an unfamiliar conspecific has been reported during the two day diestrus phase (Ho et al, 2001). Rats display hyperalgesia after being exposed to a short period of mild vibration stress in late diestrus but not when they were stressed at other stages of their cycle (Devall et al, 2009; 2015). Interestingly, the stress-induced hyperalgesia (SIH) in late diestrus was prevented by short term administration during early and late diestrus of fluoxetine (FLX), using a low dose (1.75mg Kg^{-1}). FLX is classed a selective serotonin reuptake inhibitor (SSRI). However, at low concentration it has been shown to increase the brain concentration of allopregnanolone ALLO without affecting 5-HT systems (Devall et al 2015; Pinna et al, 2006; 2009). These novel findings suggested that short-term ALLO 'replacement'' had potential as a therapeutic strategy to prevent development of PMS in women.

The SIH test is a relatively unwieldy tool since several days are needed

to complete the whole test protocol (Jørom; 1988; Devall et al, 2009). We felt that the initial finding using this test should be validated in other animal models. We therefore sought to identify a higher throughput behavioural test in female rats that could detect estrous cycle differences in responsiveness to anxiogenic stress, and to determine whether blunting the rapid fall in ALLO concentration in late diestrus using sort term administration of low dose FLX, would prevent the development of symptoms.

Initially we compared responding at different stages of the estrous cycle in four different tests of anxiety-related behaviours. We then chose the most promising test to investigate the effectiveness of short-term, low dose FLX in preventing the development of anxiogenic effects during the late diestrus phase.

Tests used were:

1) the widely used fear potentiated startle test (Davis, 1990)
2) conditioned freezing to an aversive context (Curzon et al, 2009)
3) a light switch-off test in which rats in a shuttle-box move to from one compartment to the other to turn off an aversive light (Saito and Brandão, 2016)
4) recordings of 22 kHz ultrasound vocalizations (USV) emitted during acute restraint stress (Schwarting and Wohr, 2012).

Materials and Methods

Ethical statement

All experiments received formal approval (process 08.1.1547.53.3) from the Committee on Animal Research and Ethics (CEUA) of the University of São Paulo and were performed in compliance with the recommendations of the Brazilian Society for Neuroscience and Behavior, which are in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals. The number of animals used

was the minimum required to ensure statistical reliability of the data; every effort was made to minimize the risk of any animal suffering.

Animals

A total of 239 Wistar rats, from the animal facility of the University of São Paulo at Ribeirão Preto were used. Females were at least 9 weeks old, in order to ensure that they were sexually mature and undergoing regular estrous cycles. Since the behavioural tests under investigation were developed and validated in male animals, we also included groups of age matched male rats to check that males responded in the expected way in our hands. Including a group of males would also show whether there were gross sex differences in responsiveness in the tests. The animals were housed in groups of four in plastic boxes 35 x 40 x 17cm maintained at an ambient temperature of 23^{o}C on a 12h on 12h off light dark cycle with lights on at 7am. Free access to rat chow and water was available throughout. To minimize the possibility of extraneous confounding influences, all the animals were housed in the same room within our laboratory complex on delivery from the central animal facility. Experiments were carried out in adjacent rooms and only one experimenter (RMF) handled the animals. We also set strict cytological criteria for determining estrous cycle stage (Brack et al, 2006).

Vaginal Smears

All female rats went through a vaginal smearing procedure to determine the stage of their estrous cycle. A vaginal smear was taken daily in the morning (08.00-09.00h) and again following completion of each experimental test. An inoculation loop was sterilized in a flame, dipped in sterile water and then gently inserted into the vagina to gather cells, which were then smeared onto a glass slide. The smears were stained using a staining set (Panótico Rápido, Laborclin, Paraná, Brazil). Changes in the

cytological appearance of the smears were used to identify the cycle stage and to establish that the animals were cycling normally (Brack et al, 2006). The estrous cycle comprises four phases, each one with a different cell configuration: proestrus phase was characterized by an abundance of round nucleated epithelial cells; estrus phase by larger cornified squamous cells; in early diestrus an abundance of leucocytes, typically with multi-lobed nuclei was the main characteristic; late diestrus is characterized by fewer leucocytes whose nucleus has become diffuse with many cells appearing to be disintegrating. In previous studies we have found that this distinctive change in leucocyte morphology coincides with changes in functional brain status, which includes upregulation of certain $GABA_A$ receptor subunits, a change in neuronal excitability of the midbrain periaqueductal grey matter and altered responsiveness to anxiogenic drugs (Brack and Lovick, 2007; Devall et al, 2015; Lovick et al, 2005; Soares-Rachetti et al, 2016). Only animals that had completed at least two regular cycles were used for behavioural experiments. Each rat was used for only one of the 4 behavioural tests.

Fear-potentiated startle (FPS)

Apparatus: The test cage was a wire-grid cage (16.5×7.5×7.5 cm) fixed to a response platform by four thumb screws. The floor consisted of six 3.0 mm diameter stainless-steel bars spaced 1.5 cm apart. The cage and response platform were located inside a ventilated, sound-attenuating plywood chamber (64×60×40 cm). A loudspeaker located 10 cm behind the test cage delivered both the startle stimulus (100 dB, 50 ms burst of white noise) and continuous background noise (55 dB). The startle reaction of the rats generated pressure on the platform, and the analog signals were amplified, digitized and analyzed using Startle Reflex software (Med Associates, St. Albans, Vermont, USA). The startle

reaction was recorded within a time window of 100ms after the onset of the startle stimulus. Calibration of the response platform was conducted before the experiments to ensure equivalent sensitivities across sessions.

Baseline: On the first two days, the rats were placed in the test cage for a 5 min habituation period and afterwards received a total of 30 startle stimuli with an inter-stimulus interval of 30 s. Each session lasted 20 min. The startle value of the second day (the mean startle amplitude of the session) was registered and considered as the baseline value for the animal.

Training: 24 hours after establishing the baseline the rats were conditioned to associate the same experimental cage with an aversive environment. The rat was placed in the cage and 5 min later received 10 footshocks (0.4 mA, 1 s) with a variable inter-trial interval of 60–180 s. Footshocks were delivered through the floor of the training cage by a constant-current generator built with a scrambler (Albarsch Instruments, Porto Alegre, Brazil) controlled by a microprocessor (Insight Equipments, Ribeirão Preto, Brazil). Each animal was removed 5 min after the last shock and returned to its home cage. The duration of each training session was approximately 25 min.

Testing: 24 h after the training sessions the test sessions were conducted in the same cage as before, but without footshock presentation. After 5min of habituation, the rats received 30 startle stimuli (i.e. noise bursts) with a 30s inter-stimulus interval. The value of the startle was compared with the baseline startle value. An increase startle amplitude was taken to reflect increase in anxiety level. All of the experimental steps were performed in the morning between 09.00h and 12.00h.

Conditioned freezing

Apparatus*:* Two distinct contexts, context A and context B, were used for

this experimental protocol. Context A was used as a safe context. The chamber consisted on a transparent acrylic box (20 × 30 × 35 cm) with a flat stainless steel floor enclosed in a wooden sound-attenuating box containing a sound generator that produced a 1 kHz tone (72 dB; Insight Instruments, Ribeirão Preto, Brazil) and illuminated by a 40W white lamp. Context B was used for the conditioning sessions. The chamber (48×26×25 cm) had side and back walls made of black acrylic, and the ceiling and front door were made of transparent Plexiglas. The grid consisted of 36 stainless-steel rods spaced 1.5 cm apart through which footshocks could be delivered. The chamber also was enclosed in wooden sound-attenuating box with a loudspeaker and a sound generator to deliver a 1 kHz tone (72 dB; Insight Instruments, Ribeirão Preto, Brazil) and illuminated by a 15W red lamp.

Habituation: The day before of the training and test session the animals were exposed to context A (safe) for 15 min. This period of habituation was important to decrease the novelty factor of the context on the test day.

Training: Animals were placed in the context B, and after a 5 min habituation period, they were subjected to the training phase during which they received 10 footshocks (0.6 mA, 1 s) with an inter-trial interval that was varied randomly between 30 and 120 s. No explicit cue was presented between footshocks. Each animal was removed 5 min after the last shock and returned to its home cage. Training sessions lasted approximately 20 min.

Testing: 6 hours after the training the animals were re-exposed first to context A (safe) and then the same animal was exposed to context B (aversive). The time spent freezing during an 8 min period in each context was recorded by an observer. Freezing was operationally defined as the

total absence of movement of the animal, with the exception of movements related to respiration, for a period of at least 6s. An increase in time spent freezing was taken to represent an increase in fear. The testing session was performed in the afternoon between 13.00-15.00h. Both chambers were cleaned with 20% alcohol after each experiment.

Light switch-off response (SOR)

The experimental chamber consisted of a two-compartment shuttle box (30 cm × 25 cm × 25 cm; Insight Equipment, Ribeirão Preto, Brazil). The side and back walls of the chamber were made of black Plexiglas while the front and ceiling were made of transparent Plexiglas. The experimental chamber was equipped with a grid floor (15 x 2.0 mm diameter stainless-steel rods spaced 1.2 mm apart). The shuttle behavior of the animals was measured during the session by counting the number of times the floor tipped a fulcrum in the shuttle box as the rat moved from one side to the other. The chamber was illuminated by two 40W light bulbs, one in the center of each side of the rear wall of the chamber 12 cm from the floor, which generated a light intensity of approximately 120 lux measured at the floor level of the cage. The lights turned on and off silently. The light was switched off automatically when the rat moved to the opposite side of the box and remained lit in the absence of this response. The experimental chamber was located within a small, ventilated sound-attenuating box (50 cm × 40 cm × 35 cm). The behavior of the animals during the test sessions was recorded by a video camera (Everfocus, Duarte, California, USA) positioned in the lateral wall of the observation chamber, thereby allowing all behaviours to be monitored. The video signal was relayed via a closed circuit to a monitor located in an adjacent room.

Procedure. Each animal was placed inside the unlit shuttle box and allowed 5 min to habituate to the experimental context before the beginning of the session. The animal was then exposed to the light for 20s. Whenever the rat passed from one side of the box to the other whilst the light was on (i.e. within 20 s), this response switched the light off (SOR). Successive trials were separated by an interval of equal duration in which the light remained switched off. The presentation and sequencing of the light stimuli were controlled by the manufacturer's software (*Esquiva Ativa*; Insight Equipments, Ribeirão Preto, Brazil), which also collected data on the number of shuttling responses during the light and dark events of the test in blocks of 10 trials (Reis et al. 2004). In the test conditions, each animal was submitted to only one session. The apparatus was cleaned with 20% alcohol between rats. The number of switch-off responses was taken as an inex of anxiety level.

22 kHz ultrasound vocalizations (USVs)

Procedure*:* The rat was placed in a restrainer (16 × 7 × 7 cm) made of steel rods spaced approximately 12 mm apart, which restricted the movement of the animal sufficiently to produce acute mild restraint stress. This restrainer was placed inside a larger, padded, echo-free (sound-attenuated) and ventilated chest (60 × 40 × 45 cm) illuminated by a 28W red light bulb located at the top of the chamber. The test sessions consisted of placing the rat inside the restrainer for 10 min during which time ultrasonic vocalisations (USVs) were recorded. The animal was then removed from the restrainer and the whole apparatus cleaned thoroughly with 20% ethanol.

For recording and analysis of USVs, an Electret ultrasound microphone (Emkay FG-3629; Avisoft Bioacoustics, Berlin, Germany) sensitive to frequencies of 1–100 kHz with a flat frequency response was positioned 40 cm above the floor. It was connected via an Avisoft UltraSoundGate

116 USB audio device (Avisoft Bioacoustics) to a personal computer, where acoustic data were displayed in real time by Avisoft Recorder (version 2.7; Avisoft Bioacoustics) with a sampling rate of 214,285 Hz in 16-bit format. For acoustical analysis, recordings were transferred to SASLab Pro (version 4.38; Avisoft Bioacoustics) and a fast Fourier transform was conducted (512 FFT- length, 100 % frame, Hamming window, and 75% time window overlap). Spectrograms were produced at a frequency resolution of 488 Hz and a time resolution of 0.512 ms. Calls were detected by an automatic threshold-based algorithm (threshold: –10 dB; start/end threshold: −20 dB) and a hold-time mechanism (hold time: 20ms) with a low cut-off frequency of 1 kHz. Call frequency and amplitude, derived from the average spectrum of each element, were determined automatically. Temporal parameters measured included total number of calls and call duration. An increase in either or both of these was taken to reflect increased anxiety level.

Drug testing

Fluoxetine hydrochloride (Sigma) was dissolved in saline. Two dosing regimens were tested. In the first one, drug (1.75 mg kg^{-1} i.p.) or saline vehicle was administered at 16:30–17:00 h on the evening of early diestrus. Testing was carried out in the early afternoon of the next day when the rats were in late diestrus. Fluoxetine (at least at higher doses than used here) raises brain ALLO concentration within an hour (Uzunov et al, 1996). Norfluoxetine, the major metabolite of FLX that is more potent than FLX itself at elevating ALLO concentration (Pinna et al; 2009), has a half-life in rat brain of around 8h following a single dose (Qu et al., 2009). Thus a single dose should be sufficient to blunt the sharp decline in ALLO in the brain as a consequence of the sharp decrease plasma

concentration of progesterone during early/late diestrus (Butcher et al, 1974).

The second dosing protocol was based on one we had found previously to be effective in preventing vibration stress-evoked hyperalgesia in late diestrus (Devall et al, 2015). Fluoxetine was given in the late afternoon of early diestrus with a second 'top up' injection the following morning 1 h prior to beginning behavioral testing. This dosing protocol was also followed for the control group, which received saline.

Statistical analysis

Data are presented as mean ± standard error of mean (SEM). Two-way repeated-measures ANOVA was carried out on the data using GraphPad Prism 7.0 (GraphPad Software, Inc., USA). The factor group refers to males and females in different stages of the estrous cycle and the factor condition refers to trial-type (noise alone or context + noise) in the FPS, safe and aversive context in the freezing test, blocks 1 to 4 in the light switch-off test and the recorded USV frequencies for the acute restraint stress, respectively. Post hoc differences between group means were evaluated using the Bonferroni or Tukey post hoc tests. $P < 0.05$ was considered significant.

RESULTS

Fear-potentiated startle (FPS)

Male and female rats at all stages of the estrous cycle displayed a similar baseline startle response, which increased in amplitude after being conditioned to the fearful environment. Figure 1A shows the startle amplitude in male (n=15) and female rats separated into groups according to the estrous cycle: P (n = 10), E (n =10), ED (n =10) and in LD (n = 10). Two-way repeated-measures ANOVA showed significant effects of trial

type ($F_{1,50} = 8.71$, $p < 0.05$) but no significant effect of groups ($F_{4,50} = 0.57$, $p > 0.05$) and no group $\times$ trial type interaction ($F_{4,50} = 0.15$, $p > 0.05$). The Bonferroni post hoc test revealed that compared to baseline trials, there was an overall enhancement of the startle response in male and females at all stages of their cycle in the trials conducted in the aversive context.

Conditioned freezing to context

Male and female rats at each stage of their cycle (n=8 for all groups) showed similar durations of freezing in the baseline condition and each group displayed an increase in freezing in the aversive context (Fig. 1B). Two-way repeated-measures ANOVA revealed a significant effect of trial type ($F_{1,35} = 78.57$, $p < 0.05$), but no significant effect of groups ($F_{4,35} = 2.12$, $p > 0.05$) and no groups $\times$ trial type interaction ($F_{4,35} = 0.58$, $p > 0.05$). The Bonferroni post hoc test revealed an overall enhancement of the freezing response in the aversive context trials compared with baseline trials in all groups tested.

Light switch-off responses (SOR)

Male rats and females in each stage of estrous cycle were submitted to the switch-off procedure (n= 9-10 per group). For analysis, the data was sub-divided by sex and cycle stage into 4 blocks of 10 consecutive trials (B1-B4). Fig 1C shows the number of shuttles (SORs) made in response to 40 x 20s presentations of the light. At the beginning of the test males and females in all stages of their cycle made a similar number of shuttles from light to dark. With the exception of rats in late diestrus, males and females showed a progressive decrease in the number of responses made in subsequent blocks of 10 trials. Two-way repeated-measures

ANOVA revealed a significant effect of blocks ($F_{3,126} = 10.71$, $p < 0.05$), groups (males and estrous cycle stages) ($F_{4,42} = 3.05$, $p < 0.05$) and groups $\times$ block interaction ($F_{12,126} = 2.10$, $p < 0.05$). The Bonferroni post hoc test revealed that, except for rats in late diestrus and to a lesser extent the proestrus phase, the number of SORs was reduced in the final block of the test (B4) compared with the beginning of the test (B1).

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Ultrasound vocalizations (USVs)

The number of USVs emitted during isolation restraint was measured in groups of male rats and females in each stage of the estrous cycle (Figure 1D). In male rats the number of USVs in the 20-22KHz range was greater than at other frequencies (Fig 1D). In contrast, females as a group showed a very low level of USVs at all frequencies. However, when analysed with respect to estrous cycle stage, females in late diestrus were found to make a similar number of 20-22kHz USVs to male rats (Fig 1D). Two-way repeated-measures ANOVA applied to the number of USVs emitted during the 10min test period revealed significant main effects of USV frequency ($F_{3,105} = 25.43$, $p < 0.05$), groups ($F_{4,35} = 6.95$, $p < 0.05$) and interaction between groups and USV frequencies ($F_{12,105} = 4.79$, $p < 0.05$). Tukey's post hoc test showed a significant increase in USVs in 20-22kHz range compared to 18-20kHz range only in males and females in late diestrus ($p<0.05$) (Fig 1D). There was no difference in 20-22kHz responding in females at other stages of the cycle.

In terms of duration, there was a significant main effect with respect to USV frequencies ($F_{3,105} = 8.77$, $p < 0.05$), groups ($F_{4,35} = 3.3$, $p < 0.05$) and interaction between groups and USV frequencies ($F_{12,105} = 2.8$, $p < 0.05$). Tukey's post hoc test revealed that the duration of USVs in the 20-22kHz range was significantly longer compared to 18-20kHz calls in males and females in LD but not the other cycle stages ($p<0.05$) (data not

shown).

Effect of low dose fluoxetine

We tested whether short term treatment with low dose FLX would prevent the increased sensitivity to isolation restraint stress seen in females in the late diestrus stage (n=7 per group). This series of experiments was carried out 24 months after completing the first study. In view of recent concerns about the reproducibility of data in behavioural studies and the need for ongoing test re-validation (Andrews et al, 2018), we considered it prudent to confirm the robustness of our initial findings in females before testing the effect of FLX. The second series of experiments was carried out by the same experimenter (RMF) in the same room and using the same equipment as in the initial study. In comparison to the first study carried out one year earlier, female rats in the second series of experiments emitted a greater number of USVs in all frequency ranges during isolation restraint stress. We found that rats in late diestrus made significantly more calls in the 20-22kHz range than at other stages of their estrous cycle. They also appeared to make more calls in the 18-20kHz range although the difference did not reach statistical significance (Fig 2A). Two-way repeated ANOVA applied to the number of USVs emitted during the 10min test period revealed significant main effects of USV frequencies ($F_{3,72} = 4.79$, $p < 0.05$) and borderline effects of groups ($F_{3,24} = 2.75$, $p=0.06$), but no interaction between groups and USV frequencies ($F_{9,72} = 0.92$, $p > 0.05$). Tukey's post hoc test revealed that during restraint, females in late diestrus emitted a greater number of USVs in 20-22kHz rang than at other cycle stages although the difference reached statistical significance only for females in early diestrus. No significant effects were observed in relation to the durations of USV between USV frequency ranges ($F_{3,72} = 1.61$, $p > 0.05$), groups ($F_{3,24} = 0.76$, $p > 0.05$) or

interaction between groups and USV frequencies ($F_{9,72} = 0.87$, $p > 0.05$) (data not shown).

A single dose of FLX (1.75mg Kg^{-1} ip) administered in the afternoon of early diestrus before exposure to isolation restraint stress at around 13.00h on the next day (rats now in their late diestrus phase), lead to a reduction in the number of 20-22kHz calls compared to saline-treated rats although the difference did not reach statistical significance ($p=0.2$; Fig 2B). On the other hand, when a second 'top up' dose of FLX was given in the morning of late diestrus before testing the rats later in the day, there was a significant reduction in calls made in the 20-22kHz range compared to saline-treated rats (n=9 or 10 per group). Two-way repeated ANOVA applied to the number of USVs emitted during the 10min test period revealed significant main effects of treatment ($F_{2,26} = 5.54$, $p < 0.05$), but no effects on USV frequencies ($F_{3,78} = 0.74$, $p > 0.05$) and interaction between treatment and USV frequencies ($F_{6,78} = 0.68$, $p > 0.05$). No significant effects were observed in relation to the durations of USV ($F_{3,78} = 0.16$, $p > 0.05$), treatment ($F_{2,26} = 2.36$, $p > 0.05$) or interaction between treatment and USV frequencies ($F_{6,78} = 0.49$, $p > 0.05$) (data not shown). Tukey's post hoc test revealed a significant reduction in number of 20-22kHz USVs compared to saline-treated rats ($p<0.05$). Interestingly, after 2 doses of FLX there was also a reduction in the number of calls made in the 18-20 and 22-24kHz range following two doses of FLX.

Discussion

In this study, we compared responses of male and female rats in 4 behavioural tests of anxiety-related behavior. We were particularly interested to establish whether responsiveness of females was affected by estrous cycle stage. In tests using conditioned fear paradigms (fear

potentiated startle and conditioned fear to context), females as a group responded in the same way as males and we were unable to detect any difference in responsiveness at different stages of the estrous cycle. Our findings are in agreement with previous reports of similar experiments using Wistar and Lewis rats (Cossio et al 2016; Maeng et al., 2015; Pryce et al, 1999; Zhao et al, 2018). On the other hand, they contrast with results of other studies using these tests in which males were reported to be more responsive than females (de Jongh et al, 2005; Gresack et al. 2009; Maren et al, 1994; Petterson et al, 1994). This might reflect the rather different protocols as well as strains of rat used in the latter studies.

In contrast to the tests evoking conditioned fear, clear estrous cycle linked differences were detected in performance in both the light switch-off and isolation restraint-induced ultrasound vocalisation tests. In the light switch-off test rats have the opportunity to extinguish an aversive light stimulus by moving to an unlit compartment of a shuttle box. As the experiment progresses, male rats make progressively fewer switch-off responses (SORs). This effect appears to reflect a reduction in the anxiogenic response to the light since it can be prevented by administering pro-aversive anxiogenic drugs (Saito and Brandão, 2016). In the present study males and female rats at all stages of their cycle responded similarly at the beginning of the test (Block 1). Females in late diestrus appeared to perceive the light to be aversive throughout the experiment suggesting they were more anxious than at other stages of their cycle. Rats at other stages of their cycle showed a progressive decrease in responding over successive blocks of trials although this effect was less pronounced in proestrus. During proestrus is estrogen levels are high and progesterone undergoes a spike (Butcher et al, 1974). Either of both of these factors could contribute to the behavioural profile

in proestrus.

In line with these findings robust estrous cycle-linked differences were also displayed by females subjected to isolation restraint stress. Male rats exposed to stressful aversive behavioural situations emit 22kHz calls (Brudzynski, 2015; Portfors, 2007). Males in our experiments emitted 22kHz USVs during isolation restraint, suggesting they found the experience aversive whereas the females as a group emitted far fewer calls than males, suggesting that they were less stressed by the experience (Inagaki and Mori, 2015). However when estrous cycle stage was taken into account, emission of 22kHz calls by females in late diestrus was some 3-fold higher than at other stages of the cycle, and reached the same level of responding as males. This suggests that the rats in late diestrus were much more stressed by isolation restraint in this phase than at other stages of their cycle.

It is interesting to consider why female rats behaved so differently in four behavioural tests, all of which evoked a degree of fear and/or anxiety. One possibility relates to the level of anxiety induced by the different tests. The conditioned fear paradigms (conditioned fear to context and fear-potentiated startle), both involve subjecting the animal to footshocks, which are overtly nociceptive. Reports of changes in pain threshold during the estrous cycle are equivocal (Devall et al, 2009; Gustafsson et al, 2011; Martinez-Gomez et al, 1994; Terner et al, 2005) and it is questionable whether subtle changes in threshold would be sufficient to influence the level of fear engendered by the reinforcing footshock stimulus during the conditioning stage. Even if there are estrous cycle linked differences in nociception, the level of fear evoked by the tests using footshock at all stages of the estrous cycle is likely to be much higher than evoked by the

light switch-off and restraint-induced ultrasonic vocalization tests. These are more naturalistic, ethologically relevant tests based on the innate fear of rodents for brightly-lit areas and the fear/anxiety engendered by being isolated and confined in a restricted space. It is important to note that our restraining cage does not impose the same level of distress on the animal as the complete immobilisation imposed in other studies, which is highly aversive (e.g. Costa et al., 2005; Gameiro et al., 2006). We propose that if the level of anxiety induced by a procedure is relatively low, as in the light switch-off and ultrasound vocalisation tests used in the present study, or indeed the mild vibration stress used in previous studies (Devall et al, 2015) the behaviour of female rats can be influenced by the estrous cycle. On the other hand, high levels of fear engendered by footshock, may override any estrous cycle-linked influences which are revealed under more ethologically relevant testing situations.

Progress in psychobiology is dependent on the development of appropriate robust animal models (Rodgers, 1997; Ennaceur and Chazot, 2016; Stanford, 2017), never more so than in relation to development of sex-specific pharmacology for treatment of affective disorders in women. In women with PMS, the menstrual cycle influences principally emotion but has limited effect on cognitive function (Sundström-Poromaa, 2018). The adverse symptoms experienced in the late luteal (premenstrual) phase may be considered an inappropriate over-reaction to everyday psychological stressors that at other stages of their cycle do not trigger an adverse response. The isolation restraint stress test in female rats is ethologically relevant since it is based on the innate fear of rats to being confined and isolated and evokes an unconditioned emotional response (USVs). The test is sensitive to estrous cycle stage; it requires only one test session, which can be accomplished rapidly (10min) and data

collection is fully automated, thereby favouring relatively high throughput. The test appears to fulfil our criteria for a rodent model for developing potential new pharmacological strategies for treating premenstrual dysphorias in women.

We were concerned by the differences in baseline level of responding in the two sets of experiments measuring USVs that were carried out a year apart. Reproducibility in behavioural testing in rodents is a major concern since both within- and between-lab differences are not uncommon (Andrews et al, 2018; Gulinello et al, 2018; Hogg 1996). As far as we can ascertain, nothing had changed in the experimental setting. Equipment, environment and experimenter and time of year were the same. However, our rats were obtained from the University's central breeding facility and it is possible that some subtle change within that environment may have been responsible. Another possibility is subtle changes in the environment within our local Departmental animal house where rats are housed for several weeks prior to and during the experimental period. Differences in the proportions and overall numbers of male and female rats housed there, or the number of visits to the room by staff from other research groups could have had an impact. Exposure of male rats to nociceptive stress (footshocks) lead to a long-lasting increase in numbers of 22kHz USVs tested one week later (Wöhr et al, 2005). Although any stress experienced by our female rats is likely to have been far more subtle, these findings suggest that USVs may be a sensitive indicator of long term changes. However, we are reassured that despite differences in baseline, the phenotype in terms of estrous cycle and USVs was maintained. In the second series of experiments, there was a tendency towards increased responsiveness in late diestrus in the 18-20kHz range so that responsiveness in late diestrus did not differ between these two groups, as they had done in the first study. It is worth noting that the so

called 22kHz calls considered to be indicators of anxiety/distress range from 18-32kHz (Sanchez, 2003; Schwarting and Wohr, 2012). It may be that if rats are more alert or anxious for some reason, as in our second study (see discussion page 20-21) their USV calls in response to isolation restraint generalised to include the 18-20kHz range. Our experience highlights the necessity for replication (as done in this study) to check baseline data between studies.

Notwithstanding these confounding factors, the results show clearly that the development of increased anxiety (enhanced 20-22kHz calls) in late diestrus could be prevented by short term treatment with low dose FLX. It is worth noting that FLX has not been reported to influence locomotor activity in female rats, even at doses higher than used in the present study (Fernandez-Guasti et al, 2017) and presumably would not interfere with rats' ability to vocalise. FLX increases brain concentration of ALLO by inhibiting its oxidation to 5α-dihydroprogesterone by a microsomal dehydrogenase (Fry et al, 2014). Based on the long half-life of norfluoxetine, the active metabolite of FLX (Qu et al 2009), we aimed to time dosing to offset the sharp fall in brain ALLO concentration that occurs during late diestrus so that brain concentration of the neurosteroid would decline more gradually (Devall et al, 2015; Pinna et al, 2009). In studies in which progesterone (and hence ALLO) concentrations have been manipulated artificially, the anxiogenic effect of rapid withdrawal from progesterone was ameliorated by withdrawing the steroid gradually (Doornbos et al, 2009). We have shown previously that increased anxiety-like behaviour in late diestrus, when progesterone and hence allopregnanolone levels are falling steeply, corresponds with increased expression of $GABA_A$ receptor subunits and increased excitability of circuitry in parts of the brain associated with mediating fear and anxiety-like behaviours (Griffiths and Lovick, 2005**;** Brack and Lovick, 2007; Devall

et al, 2015; Lovick et al., 2005). We reasoned that short-term administration of low dose FLX in diestrus should, by raising brain concentration of ALLO via its steroid stimulating effect, offset these effects of the natural sharp fall in ALLO. Our present results support this hypothesis and confirm our earlier finding using a different behavioural test (Devall et al, 2015). Further experimentation is required to provide more direct evidence regarding the mechanism.

The steroid stimulating effect of FLX is mediated by its metabolite norfluoxetine, which has a long half-life (Qu et al, 2009). Based on this information we had reasoned that a single dose of FLX given approximately 15h before behavioural testing might be sufficient to blunt the natural sharp decline in ALLO concentration. However, a single dose produced only modest (non-significant) reduction in isolation stress-evoked USVs in late diestrus. The steroid stimulating effect of FLX may therefore have been shorter lasting than expected, based on its reported half-life (Qu et al 2009). On the other hand, administration of FLX in the late afternoon of early diestrus, with a top up dose on the morning of late diestrus, completely prevented the development of the anxiogenic response in late diestrus. Our finding that a second dose of ALLO was required to prevent completely the increase in USVs during late diestrus does however, raise the question of whether this single dose on its own would have been effective. ALLO has been reported to be anxiolytic at pharmacological doses (e.g. Brot et al, 1997; Rogers and Johnson, 1998). However, at the physiological concentrations likely to be realized by the steroid-stimulating effect of FLX, it is anxiogenic (Andréen et al, 2009; Miczek et al, 2003, Gulinello and Smith, 2003). It seems most unlikely therefore that the anxiolytic effect in late diestrus reflects an acute response to the second dose of FLX. Even so, experiments to address this point specifically need to be carried out. It is also

worth noting that in a previous study low dose FLX administered at other stages of the cycle had no effect on anxiety-like behaviour (Devall et al, 2015), which would have been expected if its effects were due to a direct anxiogenic (or anxiolytic action).

In summary, these initial findings show that low dose FLX below the threshold for activating serotonergic systems, is able to prevent the increased responsiveness to acute stress that characterises the late diestrus phase of the estrous cycle in female rats. Fluoxetine and other selective serotonin reuptake inhibitors have become the drugs of choice for treatment of premenstrual dysphorias in women, yet their mechanism of action is incompletely understood and perhaps surprisingly, both continuous dosing or intermittent dosing during the luteal phase appear to be equally effective (Majoribanks et al, 2013). The results of the present study suggest that at last part of their effectiveness may be due to their steroid-stimulating properties. Low doses of FLX taken during the late luteal phase as symptoms appear, should lead to a positive therapeutic outcome without the risk of adverse side effects of higher doses commonly used.

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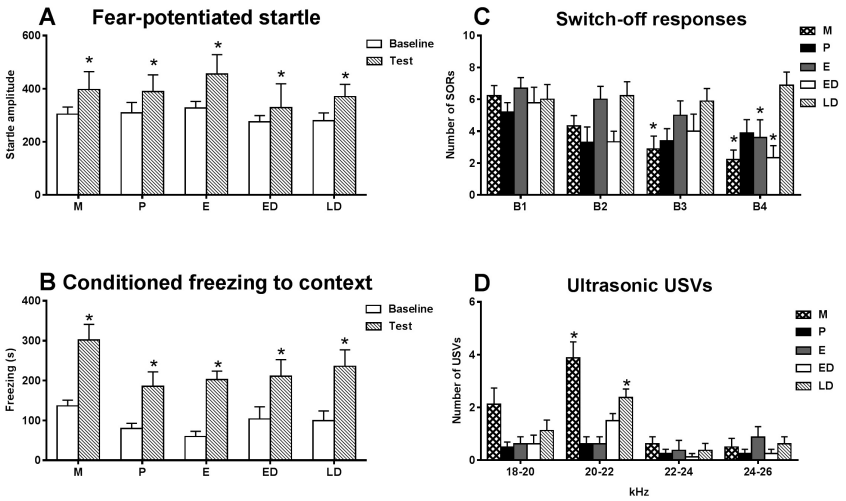

Fig.1. Responsiveness of male rats (M) and female rats at different stages of the estrous cycle in 4 different behavioural tests. Proestrus (P). Estrus (E). Early diestrus (ED). Late diestrus (LD). Results are expressed as mean ± SEM and subjected to two-way repeated measure ANOVA followed by Tukey's post hoc test.

A. Influence of the estrous cycle on the mean amplitude of startle response (arbitrary units) evoked by noise before and after exposure to aversive footshocks. * $p < 0.05$, compared with noise-alone trials in the same group (females: n = 10 per cycle stage; males: n=15).

B. Duration of freezing elicited by re-exposure of rats to a safe context and to a context in which they had previously received aversive footshocks. * $p < 0.05$, compared with baseline trials in the same group; n = 8 for all groups.

C. Number of crossings in the light (switch-off responses, SORs) made during 40 presentations of the light stimulus. Data subdivided into 4 blocks of 10 presentations (B1-B4). N=9-10 per group. * significantly different from B1 in the same group, $p< 0.05$.

D. Number of vocalizations (USVs) emitted at ultrasound frequencies (18-26 kHz) during 10 min of isolation restraint. * $p < 0.05$ in comparison to

the 18-20 kHz band for the same group used as a control. n = 8 in all groups.

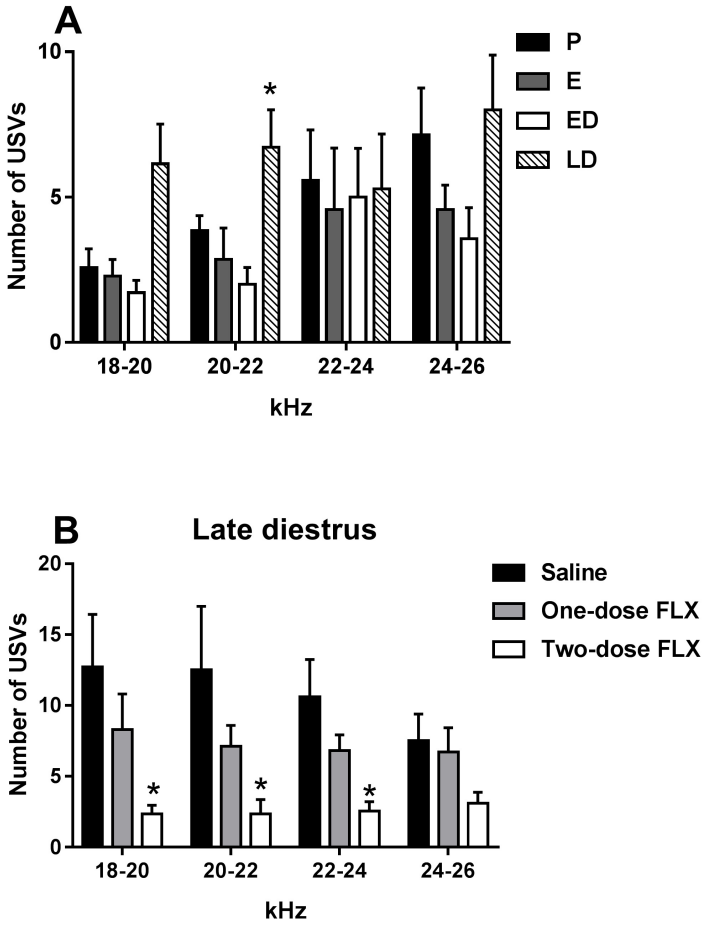

Fig 2. A. Number of USVs emitted during 10min isolation restraint stress in rats at different stages of the estrous cycle. N= 7 per group. * significantly different from early diestrus stage of the cycle in the 20-22 kHz range ($p<0.05$, Tukey's post hoc test). B. Effect of fluoxetine or saline on number of USVs emitted during isolation restraint stress in late diestrus. One dose: 1.75 mg kg^{-1} i.p. given in the afternoon of early diestrus; Two dose: a second dose given in the morning of late diestrus. * significantly different from saline-treated rats at the same frequency.